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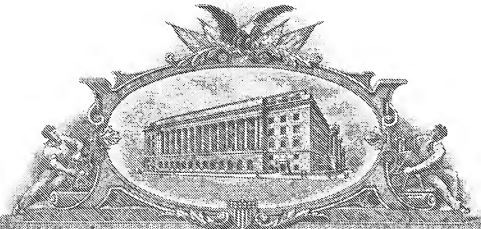

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THIS IS A REQUEST FOR FILING A PROVISIONAL APPLICATION FOR PATENT UNDER 37 C.F.R. § 1.53(C).

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TITLE OF THE INVENTION (280 characters max)

ANTHRAX ANTIGENS AND METHODS OF USE

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ANTHRAX ANTIGENS AND METHODS OF USE

BACKGROUND OF THE INVENTION

[001] Anthrax is a highly lethal infectious disease caused by *Bacillus anthracis*, a spore-forming, gram-positive bacterium. In its most lethal form, anthrax spores are inhaled and germinate inside alveolar macrophages which transport the bacterium from the lung to the host circulatory system. Once release from the macrophages, *B. anthracis* replicates extracellularly, causing flu-like symptoms followed by massive hypotension and pulmonary edema.

[002] Two major *B. anthracis* virulence factors are plasmids, pXO1 and pXO2. pXO2 encodes poly-γ-D-glutamic acid (PGA), which is a major component of the bacterium's capsule but is weakly immunogenic. PGA is also an antiphagocytic, *i.e.*, unencapsulated strains are readily phagocytized and virtually avirulent. pXO1 encodes the protein subunits of the secreted exotoxins, lethal toxin (LeTx) and Edema Toxin (EdTx). Each exotoxin consists of A and B subunits, which are both required to produce a functional toxin. The A subunit consists of either edema factor (EFa), an adenyl cyclase, to form EdTx or lethal factor (LFa), a metalloproteinase, to form LeTx. The B subunit for both exotoxins consists of protective antigen (PA), which binds to the anthrax toxin cellular receptor. In the presence of furin-like proteases, a 20 kDa piece is removed from the full length PA molecule (83 kDa), forming PA_{63} (63 kDa) revealing binding sites for interaction with other PA_{63} molecules to form heptamers (PA_7) and for interaction with A subunits LF and/or EF to form the major functional exotoxins, LeTx and/or EdTx.

[003] The current human anthrax vaccine is an aluminum hydroxide-adsorbed supernatant from fermentor cultures of a toxigenic non-encapsulated strain of *B. anthracis* V770-NPI-R. The vaccine is expensive to produce, requires repeated doses, and causes local pain with edema and erythema. The course of immunization consists of three subcutaneous injections 2 weeks apart, three injections 6 months apart, and an annual injection as long as the individual remains at risk. Recent events have demonstrated the potential use of anthrax as a bioweapon and the susceptibility of large segments of the civilian population. The disadvantages of the current vaccine make the current unfeasible for widespread administration before or after a bioterrorist attack.

[004] Thus, there is a need for effective anthrax vaccines that are suitable for widespread use. To this end, the present invention provides anthrax vaccines that target one or more of the bacterium's major virulence factors. The vaccines are formulated for administration at a mucosal surface to provide local immunity at the routes of entry of the

most lethal forms of the disease.

SUMMARY OF THE INVENTION

[005] This invention relates generally to anthrax peptides that induce an immune response to anthrax bacterium when administered to a mucosal surface. More specifically, the invention provides methods of inducing an immune response, including prophylaxis and vaccination, to anthrax bacterium by administering one or more anthrax peptides formulated with an adjuvant to a mucosal surface of a susceptible individual. The immune response induced by the anthrax peptides preferably ameliorates or prevents anthrax disease. Therefore, the compositions of the invention find use as immunogens, including, vaccines and therapeutic agents.

[006] In one embodiment, the invention provides a composition comprising an anthrax peptide and a mucosal adjuvant.

[007] In a preferred embodiment, the composition comprises an anthrax peptide of LeTx and/or EdTx, such as, PA, LF, EF, or immunogenic fragments thereof. In another preferred embodiment, the composition comprises an anthrax polypeptide of PGA or immunogenic fragments thereof. In another preferred embodiment, the composition comprises a combination of anthrax peptides or immunogenic fragments thereof.

[008] In one embodiment, the adjuvant of the composition is a mucosal adjuvant. In a preferred embodiment, the mucosal adjuvant comprises an invasin protein of a gram-negative bacteria, including but not limited to, a Shigella or Escherichia invasin protein. In another preferred embodiment, the mucosal adjuvant is an Invaplex (IPX). In another preferred embodiment, the adjuvant comprises an immunogenic component of a gram-negative bacterial cell wall, including but not limited to, lipid A. In another preferred embodiment the adjuvant comprises monophosphoryl lipid A (MPL) and/or synthetic trehalose dicorynomycolate (TDM).

[009] In other aspects, the invention provides methods of inducing an immune response to anthrax bacterium comprising administering a composition of the invention to a subject. In one embodiment, the immune response is to an anthrax peptide. In a preferred embodiment, the immune response is to a LeTx peptide or PGA. Preferably, the immune response protects or ameliorates the subject from anthrax disease. Thus, the invention provides methods of treating or protecting anthrax disease.

[010] In another aspect, the invention provides antibodies that find use as immunotherapeutics.

BRIEF DESCRIPTION OF THE DRAWINGS

[011] FIG. 1 shows ELISA results for sera collected at 4 weeks (Left) and 8 weeks (Right) following initial vaccination (Example 1). The value of each individual mouse is indicated by a circle. Mean values for each treatment ± standard error of the mean are indicated by bars. The PA content of immunizations is indicated in the horizontal axis labels.

[012] FIG. 2 shows survival of a lethal intravenous LeTx challenge by immunized mice (Example 1). The change in survivorship over time is indicated in hours post-challenge.

[013] FIG. 3 shows ELISA results for sera collected at 4 weeks (Left) and 8 weeks (Right) following the initial vaccination (Example 2). The value of each individual mouse is indicated by a circle. Mean values for each treatment ± standard error of the mean are indicated by bars. The PA content of immunizations is indicated in the horizontal axis labels.

[014] FIG. 4 shows survival of a lethal intravenous LeTx challenge by immunized mice (Example 2). The change in survivorship over time is indicated in hours post-challenge. For PA-containing immunization groups, n=5. For naive and IPX groups, n=3.

[015] FIG. 5 shows the anti-PA IgG response measured at Week 3 (Example 3).

[016] FIG. 6 shows the anti-PA IgG response measured at Week 8 (Example 3).

[017] FIG. 7 shows the anti-PA IgG response measured at 8 weeks (Example 4).

[018] FIG. 8 shows the anti-Capsule IgG response measured at 8 weeks (Example 4).

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[019] The present invention is directed to the discovery that certain anthrax antigens administered to a mucosal surface of a subject induce an immune response to anthrax bacterium. Thus, the anthrax antigens find use as both a therapeutic or preventative treatment of anthrax disease.

[020] In addition to compositions, the present invention provides methods of inducing an immune response to anthrax bacterium by inducing an immune response to anthrax antigens. The immune response inhibits or interferes with the function of the anthrax antigen, thereby treating or preventing a disease symptom.

[021] In one embodiment, the methods comprise inducing an immune response to an anthrax peptide or immunogenic fragments thereof. The immune response is induced by administering an anthrax peptide and a mucosal adjuvant to a mucosal surface of a subject. The anthrax peptide is preferably an LeTx, EdTx, PGA, or BclA, including immunogenic fragments and combinations thereof. Preferably the immune response protects the subject from anthrax disease or ameliorates a disease symptom.

[022] Accordingly, the present invention provides compositions comprising an anthrax peptide and an adjuvant that induces an immune response to anthrax bacterium. By "anthrax bacterium," "*Bacillus anthracis*," and grammatical equivalents herein are meant a gram-positive rod shaped bacterium of the genus *Bacillus* that produces or is capable of

producing at least one virulence factor of anthrax disease. Thus, "anthrax bacterium" includes the gram-positive, rod-shaped bacterium and the spores formed by the bacterium and virulence factors secreted by the bacterium, including but not limited to LeTx and EdTx. The anthrax bacterium can be a wild-type strain or a genetically engineered strain that retains virulence.

[023] By "immune response" and grammatical equivalents herein are meant any response of the immune system to an antigenic stimulus, including regulatory, e.g., T-helper response, and an effector response. Thus, immune response includes a T cell response that regulates an effector function, antibody production, including but not limited to sIgA, mucosal IgA, IgG, by B cells, and cell-mediated immunity. "Immune response" includes primary and second immune responses. By "primary immune response" is meant the immune response occurring on the first exposure to an antigen. For example, in the case of antibody synthesis, after a lag or latent period of from approximately 3 to 14 days depending on the antigen and subject, specific antibodies appear in the blood. There is a peak of IgM production lasting several days followed immediately by a peak of IgG production. Antibody production ceases after several weeks, but memory cells remain in circulation. By "secondary immune response," "anamnestic response," or "booster response" are meant the immune response occurring on the second and subsequent exposures to an antigen. The secondary immune response differs from a primary immune response qualitatively or quantitatively. For example, compared to a primary antibody response and the lag period of a secondary immune response is shorter, the peak antibody titer in a secondary immune response is higher and lasts longer, IgG production predominates, the antibodies produced have a higher affinity for the antigen, and a smaller dose of the antigen is required to initiate the response.

[024] By "immune response to anthrax bacterium" and grammatical equivalents herein is an immune response that is targeted to an anthrax bacterium or a product of an anthrax bacterium. The definition includes an immune response to an anthrax bacillus, anthrax spore, or a molecule produced by an anthrax bacterium including the spore.

[025] Thus, by "neutralization," "neutralize," "neutralizing" and grammatical equivalents herein is meant to inhibit or lessen the infective capacity or ability of anthrax bacterium. In another embodiment an anthrax antibody protects a host from anthrax infection or disease, with disease being preferred.

[026] The present invention provides a variety of anthrax peptides, for example, anthrax exotoxin peptides, PGA peptides, and BclA glycopeptides. By "protein" herein is meant at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures, i.e. "analogs", such as peptoids (see Simon et al., PNAS USA 89(20):9367 (1992)) particularly when anthrax peptides are to

be administered to a patient. Thus "amino acid", or "peptide residue", as used herein means both naturally occurring and synthetic amino acids. For example, homophenylalanine, citrulline and noreleucine are considered amino acids for the purposes of the invention. "Amino acid" also includes imino acid residues such as proline and hydroxyproline. The side chain may be in either the (R) or the (S) configuration. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard in vivo degradation. In some embodiments, the amino acid linkages are gamma-linkages.

[027] By "anthrax protein," "anthrax peptide," "bacterial protein," "bacterial peptide" and grammatical equivalents herein are meant a protein having a sequence homologous or identical to an amino acid sequence deduced from an anthrax bacterium. In one embodiment, an anthrax peptide is a full length peptide as produced by an anthrax bacterium. In another embodiment an anthrax peptide is a fragment of a full-length peptide. By "fragment" and grammatical equivalents herein are meant a decrease in length by at least one amino acid residue in comparison to full length peptide as produced by an anthrax bacterium. In another embodiment, an anthrax peptide is about 5 to 50 amino acids in length. In another embodiment, an anthrax peptide is about 5 to 30 amino acids in length. In an even more preferred embodiment, an anthrax peptide is about 5 to 15 amino acids in length.

[028] Preferably "anthrax protein," "anthrax peptide," "bacterial protein," "bacterial peptide" and grammatical equivalents herein are meant a protein comprising a sequence homologous or identical to either the PA, LF, EF, PGA, or BclA peptides of anthrax bacterium, as known in the art. As used herein, a peptide is a "anthrax peptide" if the overall homology of the peptide sequence to the amino acid sequences of an anthrax peptide is preferably greater than about 75% more preferably greater than about 80%, even more preferably greater than about 85% and most preferably greater than 90%. In some embodiments the homology will be as high as about 93 to 95 or 98%. Homology in this context means sequence similarity or identity, with identity being preferred. This homology will be determined using standard techniques known in the art as described below.

[029] In a preferred embodiment, the invention provides anthrax peptides for use in a variety of applications, as outlined below. By "anthrax peptide" and grammatical equivalents herein includes both full length anthrax peptides, anthrax peptides fragments and synthetic peptides. Thus, the anthrax peptides may be shorter or longer than the naturally occurring amino acid sequences. In a preferred embodiment, included within the definition of anthrax peptide are proteins and portions or fragments of the proteins encoded by anthrax bacterium. Peptides are considered anthrax peptides if they possess one or more of the following: a) they exhibit the ability to block binding of an antibody to anthrax bacterium or protein; b) they exhibit the ability to block binding of an anthrax protein to a host cell or to

another anthrax protein or peptide; c) they induce antibody cross-reactive with an anthrax protein; d) they exhibit at least one biological activity of a naturally-occurring anthrax protein; e) they have at least the indicated homology; f) they induce an immune response to an anthrax protein or bacterium; or g) they induce a protective or therapeutic immune response to anthrax disease. In a preferred embodiment, an anthrax protein exhibits two or more of these characteristics. In addition, preferred embodiments include anthrax peptides that share at least one antigenic epitope with a naturally occurring protein.

[030] Thus, in a preferred embodiment an "anthrax peptide" includes a peptide that induces an antibody that binds to an amino acid sequence deduced from an anthrax bacterium nucleic acid. As known in the art, antibodies recognize either linear or conformational epitopes. By "epitope," "antigenic determinant," and grammatical equivalents herein are meant a region of an antigen or immunogen that is specifically bound by an antibody. Accordingly, an epitope can be linear or conformational. By "linear epitope" herein is meant a epitope comprising a sequence of at least about 5 and not more than about 20 amino acids connected in a linear fashion, which amino acids, by themselves or as part of a larger sequence, bind to an antibody generated in response to such sequence. By "conformational epitope" is meant an epitope whose three dimensional or tertiary structure binds to an antibody. Generally but not uniformly, amino acids that comprise a conformational epitope do not comprise a linear sequence of a protein's primary structure. Thus, a conformational epitope may be shared by proteins having non-homologous linear amino acid sequences. Rather, a conformational epitope is shared because the tertiary structure recognized by an antibody is shared between the proteins. A peptide or protein that mimics the conformational structure of a naturally occurring anthrax protein is a mimotope. By "mimotope" and grammatical equivalents herein are meant a compound that mimics, resembles, copies, or imitates the structure of an epitope and induces, provokes, or reacts with the immune response to the epitope. For example, in one embodiment, a mimotope is a protein mimotope that resembles, copies, or imitates the structure of the epitope. In other examples, a mimotope of a protein epitope is a carbohydrate, a nucleic acid, an organic compound, or protein, or a derivative or analog of any one of these. Mimotopes are identified by various assays, including but not limited to screening a phage expression library, a cell expression library, a chemical library as known in the art. In a preferred embodiment, the anthrax peptides are PA, LF, EF, PGA, BclA peptides and immunogenic fragments thereof.

[031] When an anthrax peptide is used to generate anthrax antibodies, the anthrax peptide must share at least one epitope or determinant with the full length anthrax protein. By "epitope" or "determinant" herein is meant a portion of a protein which will generate and/or bind an antibody. Accordingly, epitopes or determinants may be linear or conformational as described herein. In most instances, antibodies made to a smaller anthrax peptide bind to the full length protein. In a preferred embodiment, the epitope is unique; that

is, antibodies generated to a unique epitope show little or no cross-reactivity to other proteins. In an alternative embodiment, the epitope generates antibodies that recognize complexes of anthrax proteins, such as, the associated A and B subunits which form anthrax exotoxin or the PA heptamer.

[032] Also included within the definition of anthrax peptides of the present invention are amino acid sequence variants. These variants fall into one or more of three classes: substitutional, insertional or deletional variants. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding an anthrax peptide, using cassette or PCR mutagenesis or other techniques well known in the art, to produce DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture as outlined above. However, variant anthrax peptides having up to about 100-150 residues may be prepared by in vitro synthesis using established techniques. Amino acid sequence variants are characterized by the predetermined nature of the variation, a feature that sets them apart from naturally occurring variation of the anthrax peptide amino acid sequence. The variants typically exhibit the same qualitative biological activity as the naturally occurring analogue, although variants can also be selected which have modified characteristics as will be more fully outlined below.

[033] While the site or region for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed anthrax peptide variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example, M13 primer mutagenesis and PCR mutagenesis. Screening of the mutants is done using assays of anthrax peptide activities.

[034] Amino acid substitutions are typically of single residues; insertions usually will be on the order of from about 1 to 20 amino acids, although considerably larger insertions may be tolerated. Deletions range from about 1 to about 20 residues, although in some cases deletions may be much larger.

[035] Substitutions, deletions, insertions or any combination thereof may be used to arrive at a final derivative. Generally these changes are done on a few amino acids to minimize the alteration of the molecule. However, larger changes may be tolerated in certain circumstances. When small alterations in the characteristics of the anthrax peptide are desired, substitutions are generally made in accordance with the following chart:

Chart I

Original Residue	Exemplary Substitutions
Ala	Ser
Arg	Lys

Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Ile
Phe	Met, Leu, Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

[036] Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those shown in Chart I. For example, substitutions may be made which more significantly affect: the structure of the polypeptide backbone in the area of the alteration, for example the alpha-helical or beta-sheet structure; the charge or hydrophobicity of the molecule at the target site; or the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the polypeptide's properties are those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g. lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g. glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g. glycine.

[037] The variants typically exhibit the same qualitative biological activity and will elicit the same immune response as the naturally-occurring analogue, although variants also are selected to modify the characteristics of the anthrax peptide as needed. Alternatively, the variant may be designed such that the biological activity of the anthrax peptide is altered.

[038] Covalent modifications of anthrax peptides are included within the scope of this invention, particularly for screening assays or therapeutic uses. One type of covalent modification includes reacting targeted amino acid residues of anthrax peptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N-or C-terminal residues of an anthrax peptide. Derivatization with bifunctional agents is useful, for instance, for crosslinking anthrax peptide to a water-insoluble support matrix or surface for use in the methods described below, or for in vivo stability. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional

imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate.

[039] Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the "-amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

[040] In addition, modifications such as derivitization with polyethylene glycols (and other glycols) to increase the in vivo stability half-life are also included.

[041] Anthrax peptides of the present invention may also be modified in a way to form chimeric molecules comprising an anthrax peptide fused to another, heterologous polypeptide, for example another anthrax peptide, or amino acid sequence. In a preferred embodiment the anthrax peptide may be linked to adjuvants or other molecules to increase the immune response to the peptide. In an additional embodiment, such a chimeric molecule comprises a fusion of an anthrax peptide with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino-or carboxyl-terminus of the peptide. The presence of such epitope-tagged forms of an anthrax peptide can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the anthrax peptide to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag; this is also useful for binding the anthrax peptide to a support for heterogeneous screening methods. Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)].

[042] By "nucleic acid," "oligonucleotide," and grammatical equivalents herein are meant at least two nucleotides covalently linked together.

[043] The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence.

[044] By "anthrax nucleic acid," and grammatical equivalents herein are meant a nucleic acid comprising a sequence homologous or identical to all of part the anthrax bacterium genome or an extrachromosomal element that encodes an thrax peptide as defined herein.

[045] As is known in the art, a number of different programs can be used to identify whether a protein (or nucleic acid as discussed herein) has sequence identity or similarity to a known sequence. Sequence identity and/or similarity is determined using standard techniques known in the art, including, but not limited to, the local sequence identity algorithm of Smith and Waterman, Adv. Appl. Math. 2:482 (1981), by the sequence identity alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, WI), the Best Fit sequence program described by Devereux et al., Nucl. Acid Res. 12:387-395 (1984), preferably using the default settings, or by inspection. Preferably, percent identity is calculated by FastDB based upon the following parameters: mismatch penalty of 1; gap penalty of 1; gap size penalty of 0.33; and joining penalty of 30, "Current Methods in Sequence Comparison and Analysis" in Macromolecule Sequencing and Synthesis, Selected Methods and Applications, pp 127-149, Alan R. Liss, Inc. (1988).

[046] An example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle, J. Mol. Evol. 35:351-360 (1987); the method is similar to that described by Higgins and Sharp CABIOS 5:151-153 (1989). Useful PILEUP parameters including a default gap weight of 3.00, a default gap length weight of 0.10, and weighted end gaps.

[047] Another example of a useful algorithm is the BLAST algorithm, described in Altschul et al., J. Mol. Biol. 215, 403-410, (1990) and Karlin et al., Proc. Natl. Acad. Sci. USA 90:5873-5787 (1993). A particularly useful BLAST program is the WU-BLAST-2 program which was obtained from Altschul et al., Methods in Enzymology, 266: 460-480 (1996); http://blast.wustl/edu/blast/ README.html). WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span =1, overlap fraction = 0.125, word threshold (T) = 11. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched; however, the values may be adjusted to increase sensitivity.

[048] An additional useful algorithm is gapped BLAST as reported by Altschul et al. Nucleic Acids Res. 25:3389-3402. Gapped BLAST uses BLOSUM-62 substitution scores; threshold T parameter set to 9; the two-hit method to trigger ungapped extensions; charges gap lengths of k a cost of 10+k; Xu set to 16, and Xg set to 40 for database search stage and to 67 for the output stage of the algorithms. Gapped alignments are triggered by a score corresponding to ~22 bits.

[049] A percent amino acid sequence identity value is determined by the number of matching identical residues divided by the total number of residues of the "longer" sequence in the aligned region. The "longer" sequence is the one having the most actual residues in the aligned region (gaps introduced by WU-Blast-2 to maximize the alignment score are ignored).

[050] The alignment may include the introduction of gaps in the sequences to be aligned. In addition, it is understood that in one embodiment, the percentage of sequence identity will be determined based on the number of identical amino acids in relation to the total number of amino acids. Thus, for example, sequence identity will be determined using the number of amino acids in the shorter sequence. In percent identity calculations relative weight is not assigned to various manifestations of sequence variation, such as, insertions, deletions, substitutions, etc.

[051] In one embodiment, only identities are scored positively (+1) and all forms of sequence variation including gaps are assigned a value of "0", which obviates the need for a weighted scale or parameters for sequence similarity calculations. Percent sequence identity can be calculated, for example, by dividing the number of matching identical residues by the total number of residues of the "shorter" sequence in the aligned region and multiplying by 100. The "longer" sequence is the one having the most actual residues in the aligned region.

[052] By "antibody" and grammatical equivalents herein are meant polyclonal and monoclonal antibody (mAb). Methods of preparation and purification of monoclonal and polyclonal antibodies are known in the art and e.g., are described in Harlow and Lane, Antibodies: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1988).

[053] By "anthrax antibody" and grammatical equivalents thereof include an antibody that binds to an anthrax peptide. The binding of an anthrax antibody to an anthrax peptide preferably prevents or treats a symptoms of anthrax disease. In another embodiment, and anthrax antibody acts as an opsonin to promote the phagocytosis of anthrax bacterium. In other embodiments, an anthrax antibody preferably inhibits the binding of anthrax toxin to a host cell. In another embodiment an anthrax antibody neutralizes anthrax bacterium or anthrax exotoxin.

[054] Anthrax antibodies usually are generated in a subject by immunization with an anthrax peptide, such as, PA, LF, EF, PGA, BclA.

[055] The terms "antibody" and "anthrax antibody," include antibody fragments and derivatives, as are known in the art, such as Fab, Fab', F(ab')2 or other antigen-binding subsequences of antibodies, such as, single chain antibodies (Fv for example), chimeric antibodies, etc., either produced by the modification of whole antibodies or those synthesized de novo using recombinant DNA technologies. The term "antibody" further comprises polyclonal antibodies and mAbs which can be agonist or antagonist antibodies as well as antibodies that have been derivatized for example with PEG as known in the art or variants as described herein.

[056] Anthrax antibodies of the invention specifically bind to anthrax proteins or peptides. By "specifically bind" herein is meant that the anthrax antibodies have a binding constant in the range of at least 10^{-4} - 10^{-6} M^{-1}, with a preferred range being 10^{-7} - 10^{-9} M^{-1}. Thus, in a preferred embodiments anthrax antibodies block the binding of a second antibody to an anthrax protein block the binding of an anthrax protein or a host cell. In an alternative embodiment, anthrax antibodies function as opsonins to facilitate phagocytosis of an anthrax bacterium. By "opsonin" and grammatical equivalents herein are meant any substance that binds to particulate antigens and induces their phagocytosis by phacocytic cells. In a preferred embodiment, opsonizing antibodies are IgM or IgG and certain complement fragments (C3b, C3d, and C4b, which become bound to the antigen during complement activation), both of which trigger phagocytosis by binding to specific cell-surface receptors, Fc receptors, C3b receptors or C3d receptors on phagocytic cells.

[057] In a preferred embodiment, an anthrax peptide of the present invention may be identified by its immunological activity, e.g., its ability to induce an immune response to an anthrax bacterium. The term "immunological activity" means the ability of an anthrax peptide to cross react with or induce an immune response to an anthrax bacterium.

[058] In a preferred embodiment, anthrax antibodies are provided. Anthrax antibodies may be polyclonal or monoclonal. In a preferred embodiment, anthrax antibodies are capable of reducing or eliminating the biological function of an anthrax protein, as is described below. That is, the addition of anthrax antibodies (either polyclonal or monoclonal) to anthrax protein may decrease or eliminate anthrax infectivity or exotoxin activity, such as, binding of an anthrax protein to a host cell. Generally, at least about a 25% decrease is preferred, with at least about 50% being particularly preferred and at least about a 95-100% decrease being especially preferred.

[059] Once made, the anthrax compositions of the invention (e.g. antibodies and peptides) find use in a number of applications. Particularly preferred are therapeutic treatments as outlined below.

[060] In a preferred embodiment, the anthrax compositions of the invention find use in the treatment of anthrax disease. "Treatment" refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down

(lessen) the targeted pathologic condition or disorder. By "host," "subject," "patient," "individual" and grammatical equivalents herein are meant those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented.

[061] In a preferred embodiment, anthrax antibody of the present invention that bind to an anthrax protein are administered to a patient in a therapeutically effective amount. By "therapeutically effective amounts" herein is meant an amount of antibody which is sufficient to ameliorate anthrax disease. This amount may be different depending on whether prophylactic or therapeutic treatment is desired. Determining the dosages and times of administration for a therapeutically effective amount are well within the skill of the ordinary person in the art. These amounts may be adjusted depending on the severity of disease or susceptibility of the patient.

[062] In a preferred embodiment, anthrax peptides of the present invention find use as vaccines and antibacterial compounds.

[063] By "vaccine" herein is meant an antigen or compound which elicits an immune response in a patient. The vaccine may be administered prophylactically, for example to a patient never previously exposed to the antigen, such that subsequent infection by anthrax bacterium is prevented. Alternatively, the vaccine may be administered therapeutically to a patient previously exposed or infected by anthrax bacterium. While infection cannot be prevented, in this case an immune response is generated which allows the patient's immune system to more effectively combat the infection. Thus, for example, there may be a decrease or lessening of a symptom associated with infection. In a preferred embodiment, an anthrax vaccine comprises anthrax peptides that induce an immune response to a various types of anthrax proteins.

[064] The administration of an anthrax peptide as a vaccine is done in a variety of ways, e.g., parenteraly or mucosally, e.g., oral, nasal, rectal. Generally, the anthrax peptides can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby therapeutically effective amounts of anthrax peptide are combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation are well known in the art. Such compositions will contain pharmaceutically effective amount of anthrax peptide together with a suitable amount of vehicle in order to prepare pharmaceutically acceptable compositions for effective administration to a patient. The composition may include salts, buffers, carrier proteins such as serum albumin, targeting molecules to localize anthrax peptides at the appropriate site or tissue within the patient, and other molecules. The composition may include adjuvants as well. The formulation is chosen at the discretion of the practitioner and is dependent on the route of immunization, age and immune status of the patient, and severity of disease.

[065] In a preferred embodiment, anthrax peptides vaccines comprise a mucosal adjuvant. By "mucosal adjuvant" and grammatical equivalents herein is meant stimulator of the immune response suitable for administration at a mucosal surface, such as, oral, respiratory, nasal, with respiratory being preferred, GI, or GU tracts. In a preferred embodiment, a mucosal adjuvant is an isolated extract comprising a protein or lipid of a gram-negative bacterial cell well or outer layer, such as, an invasin protein or lipid A. Thus, in a preferred embodiment, a mucosal adjuvant is Invaplex50 or Invaplex 25. In another preferred embodiment, a mucosal adjuvant is Monophosphoryl lipid A (MPL). In another preferred embodiment the mucosal adjuvant comprises TDM (synthetic trehalose dicorynomycolate). In another embodiment, the mucosal adjuvant is cholera toxin or the adjuvant component thereof. In a preferred embodiment, compositions comprising anthrax peptide and cholera toxin mucosal adjuvant induce protective immunity against anthrax exotoxin or anthrax disease. For example protection against anthrax exotoxin or disease is neutralization of anthrax exotoxin in vivo.

[066] Invaplex and methods of use are described in U.S. Patent Nos. 6,245,892, 6,277,379, 6,680374, and PCT Publication No. WO02/094190, all four of which are expressly incorporated by reference in their entirely. An Invaplex preferably finds use as a mucosal adjuvant to induce a mucosal immune response to anthrax peptides, such as, IgA. In another preferred embodiment, anthrax peptide vaccines comprise and an M cell targeting ligand. By "M cell targeting ligand" and grammatical equivalents herein are meant a compound that binds to an receptor on M cells. In this embodiment, the M cell targeting ligand preferably is selected from the group consisting of the protein σ1 of a reovirus, or is (or is derived from) an adhesin of Salmonella or a poliovirus. In a most preferred embodiment, the M cell targeting ligand is a σ1 protein. M cell ligands target anthrax peptides to follicle associated epithelium or M cells by receptor-mediated endocytosis to induce mucosal immunity. M cell targeting ligands and methods of use are described in PCT Publication Nos. WO01/49867 and WO02/072015, both of which are expressly incorporated by reference.

[067] Where sustained-release administration of an anthrax peptide is desired in a formulation with release characteristics suitable for the treatment of any disease or disorder requiring administration of the anthrax peptide, microencapsulation of the polypeptide is contemplated. Microencapsulation of recombinant proteins for sustained release has been successfully performed with human growth hormone (rhGH), interferon- (rhIFN-), interleukin-2, and MN rgp120. Johnson et al., Nat. Med., 2:795-799 (1996); Yasuda, Biomed. Ther., 27:1221-1223 (1993); Hora et al., Bio/Technology, 8:755-758 (1990); Cleland, "Design and Production of Single Immunization Vaccines Using Polylactide Polyglycolide Microsphere Systems," in Vaccine Design: The Subunit and Adjuvant Approach, Powell and Newman, eds, (Plenum Press: New York, 1995), pp. 439-462; WO 97/03692, WO 96/40072,

WO 96/07399; and U.S. Pat. No. 5,654,010. The sustained-release formulations of polypeptides were developed using poly-lactic-coglycolic acid (PLGA) polymer due to its biocompatibility and wide range of biodegradable properties. The degradation products of PLGA, lactic and glycolic acids, can be cleared quickly within the human body. Moreover, the degradability of this polymer can be adjusted from months to years depending on its molecular weight and composition. Lewis, "Controlled release of bioactive agents from lactide/glycolide polymer," in: M. Chasin and R. Langer (Eds.), Biodegradable Polymers as Drug Delivery Systems (Marcel Dekker: New York, 1990), pp. 1-41.

[068] "Pharmaceutically acceptable salt" refers to a salt of a compound of the invention which is made with counterions understood in the art to be generally acceptable for pharmaceutical uses and which possesses the desired pharmacological activity of the parent compound. Such salts include: (1) acid addition salts, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like; or formed with organic acids such as acetic acid, propionic acid, hexanoic acid, cyclopentanepropionic acid, glycolic acid, pyruvic acid, lactic acid, malonic acid, succinic acid, malic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, 3-(4-hydroxybenzoyl) benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, 1,2-ethane-disulfonic acid, 2-hydroxyethanesulfonic acid, benzenesulfonic acid, 4-chlorobenzenesulfonic acid, 2-naphthalenesulfonic acid, 4-toluenesulfonic acid, camphorsulfonic acid, 4-methylbicyclo[2.2.2]-oct-2-ene-1-carboxylic acid, glucoheptonic acid, 3-phenylpropionic acid, trimethylacetic acid, tertiary butylacetic acid, lauryl sulfuric acid, gluconic acid, glutamic acid, hydroxynaphthoic acid, salicylic acid, stearic acid, muconic acid and the like; or (2) salts formed when an acidic proton present in the parent compound is replaced by a metal ion, e.g., an alkali metal ion, an alkaline earth ion, or an aluminum ion; or coordinates with an organic base such as ethanolamine, diethanolamine, triethanolamine, N-methylglucamine, morpholine, piperidine, dimethylamine, diethylamine and the like. Also included are salts of amino acids such as arginates and the like, and salts of organic acids like glucuronic or galacturonic acids and the like (see, e.g., Berge et al., 1977, J. Pharm. Sci. 66:1-19).

[069] "Pharmaceutically acceptable vehicle" refers to a diluent, adjuvant, excipient or carrier with which a compound of the invention is administered.

[070] "Pharmaceutically effective amount" or "therapeutically effective amount" refers to an amount sufficient to produce the desired physiological effect or amount capable of achieving the desired result, particularly for treating the disorder or disease condition, including reducing or eliminating one or more symptoms of the disorder or disease or prevention of the disease or condition. Accordingly, in a preferred embodiment, vaccines induce an immune response that reduces or eliminates one or more symptoms of anthrax disease or prevents anthrax disease or condition. Generally, this ranges from about 0.001

mg to about 1 gm, with a preferred range of about 0.05 mg. These amounts may be adjusted if adjuvants are used.

[071] In a preferred embodiment, the compositions of the invention are anti-anthrax compounds. By "anti-anthrax" and grammatical equivalents herein are meant a compound that inhibits the replication of anthrax bacterium, inhibits anthrax exotoxin, or reduces an anthrax disease symptom. Thus, an anthrax peptide may be administered prophylactically, for example to a patient never previously exposed to anthrax bacterium, such that subsequent infection by anthrax is prevented. Alternatively, anthrax peptide may be administered therapeutically to a patient previously exposed or infected by anthrax. Anthrax peptides compounds may be administered per se, but are typically formulated and administered in the form of a pharmaceutical composition. The exact composition will depend upon, among other things, the method of administration, such as orally or parenterally, and will be apparent to those of skill in the art. A wide variety of suitable pharmaceutical compositions are described, for example, in Remington's Pharmaceutical Sciences, 20th ed., 2001).

[072] Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the active compound suspended in diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as liquids, solids, granules or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, e.g., sucrose, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the active ingredient, carriers known in the art.

[073] Suitable formulations for rectal administration include, for example, suppositories, which consist of the packaged nucleic acid with a suppository base. Suitable suppository bases include natural or synthetic triglycerides or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the compound of choice with a base, including, for example, liquid triglycerides, polyethylene glycols, and paraffin hydrocarbons.

[074] Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the

formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions can be administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally. Parenteral administration, oral administration, subcutaneous administration and intravenous administration are the preferred methods of administration. A specific example of a suitable solution formulation may comprise from about 0.5-100 mg/ml compound and about 1000 mg/ml propylene glycol in water. Another specific example of a suitable solution formulation may comprise from about 0.5-100 mg/ml compound and from about 800-1000 mg/ml polyethylene glycol 400 (PEG 400) in water.

[075] A specific example of a suitable suspension formulation may include from about 0.5-30 mg/ml compound and one or more excipients selected from the group consisting of: about 200 mg/ml ethanol, about 1000 mg/ml vegetable oil (e.g., corn oil), about 600-1000 mg/ml fruit juice (e.g., grapefruit juice), about 400-800 mg/ml milk, about 0.1 mg/ml carboxymethylcellulose (or microcrystalline cellulose), about 0.5 mg/ml benzyl alcohol (or a combination of benzyl alcohol and benzalkonium chloride) and about 40-50 mM buffer, pH 7 (e.g., phosphate buffer, acetate buffer or citrate buffer or, alternatively 5% dextrose may be used in place of the buffer) in water.

[076] A specific example of a suitable liposome suspension formulation may comprise from about 0.5-30 mg/ml compound, about 100-200 mg/ml lecithin (or other phospholipid or mixture of phospholipids) and optionally about 5 mg/ml cholesterol in water. For subcutaneous administration of certain PBI compounds, a liposome suspension formulation including 5 mg/ml compound in water with 100 mg/ml lecithin and 5 mg/ml compound in water with 100 mg/ml lecithin and 5 mg/ml cholesterol provides good results.

[077] The formulations of compounds can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials. Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

[078] The pharmaceutical preparation is preferably in unit dosage form. In such form the preparation is subdivided into unit doses containing appropriate quantities of the active component. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, such as packeted tablets, capsules, and powders in vials or ampoules. Also, the unit dosage form can be a capsule, tablet, cachet, or lozenge itself, or it can be the appropriate number of any of these in packaged form. The composition can, if desired, also contain other compatible therapeutic agents, discussed in more detail, below.

[079] In therapeutic use for the treatment of anthrax infection, the anthrax compositions (e.g., antibodies and peptides) utilized in the pharmaceutical method of the invention are administered to patients diagnosed with anthrax infection at dosage levels suitable to achieve therapeutic benefit. By therapeutic benefit is meant that the

administration of compound leads to a beneficial effect in the patient over time. For example, therapeutic benefit is achieved when the anthrax bacterium titer or load in the patient is either reduced or stops increasing or a symptom or an anthrax exotoxin is reduced or stops increasing. Therapeutic benefit is also achieved if the administration of compound slows or halts altogether the onset of adverse symptoms that typically accompany anthrax infections, regardless of the anthrax bacterium or anthrax exotoxin titer or load in the patient.

[080] The anthrax peptides and/or anthrax antibodies may also be administered prophylactically in patients who are at risk of developing anthrax infection, or who have been exposed to anthrax bacterium, to prevent the development of anthrax infection or disease. For example, the anthrax peptides and/or compositions thereof may be administered to a patient likely to have been exposed to anthrax bacterium.

[081] The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes.

[082] All references cited herein are incorporated by reference.

EXAMPLES

Example 1

Intranasal Vaccination with Protective Antigen

[083] Protocol: Female Balb/c mice, 8 weeks old were immunized on days 0, 14, and day 28 with 10 µl total volume of vaccine per animal. Prior to immunization, Mice were lightly anesthetized with isofluorane to effect (2-4%), and a 5 µl/nostril bolus was delivered to each nares. Retention of the vaccine within the nares was generally good, but when sneezing occurred it was noted.

[084] Vaccine formulation: Mice were immunized with either 5 µg or 20 µg doses of PA. Lyophilized PA was obtained from List Biochemical (Cat. #171B, Lot #1712B) and reconstituted in water immediately prior to use, which brought the salt content to 5 mM HEPES/50 mM NaCl. MPL+TDM (MPL) (Sigma #M-6536, Lot #072K1313) was warmed to 40°C and resuspended in 1 mL 150 mM NaCl immediately prior to use. Invaplex 50 (IPX50) (Lot #GNGO), obtained from Edwin V. Oaks (Walter Reed Army Institute of Research (WRAIR)), was stored at –80°C and immediately prior to use was thawed on ice. Adjuvants were added to PA at 5 µl/dose just prior to use.

[085] Adjuvants: IPX50 is an ion-exchange chromatography fraction of a water extract isolated from Shigella bacteria containing the Shigella invasion complex (U.S. Patent Nos. 6,680,374, 6,277,379, 6,245,892. MPL is monophosphoryl lipid A and synthetic treholose dicorynomycolate in squalene and Tween 80.

[086] Treatment Groups: See Table 1

Table 1
Treatment Groups

Group #	Treatment	Short name	No. mice
1	MPL	MPL	3
2	Invaplex 50	IPX	3
3	5 µg PA/Invaplex 50	PA5/IPX	5
4	20 µg PA/Invaplex 50	PA20/IPX	5
5	5 µg PA/MPL + TDM	PA5/MPL	5
6	20 µg PA/MPL + TDM	PA20/MPL	5
7	Naïve	Naïve	3

[087] Sampling: Fecal and blood samples were collected from the mice prior to the initial vaccination (day 0) and on days 27 and 56 (prior to boosts).

[088] Anthrax Lethal Toxin Challenge: One week following collection of final samples, mice were subjected to an intravenous challenge with approximately 6 LD_{50} of *B. anthracis* Lethal Toxin (LeTx). PA (60 µg) and LF (30 µg) (List Biochemical; PA #171B, Lot #1712B; LF #172B, Lot #1721B) were injected via tail vein in a total volume of 100 µl PBS. Mice were observed every 15 minutes for 16 hours post-injection, and hourly for hours 20 through 36. Thereafter, mice were checked every 6-8 hours until Day 7 (168 hours). Time to death (TTD) was recorded for each mouse that died. Mice living at 168 hours were survivors (S).

[089] Serum IgG responses: Serum anti-PA IgG was measured by ELISA (Table 2. Six rows of wells in a 96 well plate (Rows C-G) were coated with 1 µg/ml PA in phosphate buffered saline (PBS) pH7.2. Rows A and B were coated with serial dilutions (1:2) of mouse IgG (Sigma I-5381), beginning at 1 µg/ml in PBS, and continuing to ~2 ng/ml in column 10, to serve as standards. Absorbances from these wells were used to plot a standard curve using SoftMax Pro software. Wells A11, A12, B11 and B12 were blanks. The serum samples were diluted from 1:100 to 1:3200 (unless otherwise noted) in PBS with 0.05% Tween 20 and 3% fetal bovine serum (FBS). Detection was via HRP-conjugated antibody (Southern Biotech) and ABTS (Pierce Cat # 37615). Each sample replicate was run on separate plates, with at least 2 analyses performed per sample. Sample absorbances were used to interpolate antibody concentration from the standard curves. Data was then exported to Excel (Microsoft®) for further analysis. If the CV (coefficient of variation, or 100*Standard Deviation/Mean) for the replicates was greater than 25, another replicate was performed. The exception to this rule was where the mean results were less than 10 µg/ml, in which case a CV of 150 was tolerated. Sample results reading above the standard curve range were deemed out of range, and were reanalyzed using a starting dilution of 1:1000.

Table 2
ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Mouse 1 1:3200	Mouse 1 1:1600	Mouse 1 1:800	Mouse 1 1:400	Mouse 1 1:200	Mouse 1 1:100	IgG 1:100	IgG 1:100				
B	Mouse 2 1:3200	Mouse 2 1:1600	Mouse 2 1:800	Mouse 2 1:400	Mouse 2 1:200	Mouse 2 1:100	IgG 1:200	IgG 1:200				
C	Mouse 3 1:3200	Mouse 3 1:1600	Mouse 3 1:800	Mouse 3 1:400	Mouse 3 1:200	Mouse 3 1:100	IgG 1:400	IgG 1:400				
D	Mouse 4 1:3200	Mouse 4 1:1600	Mouse 4 1:800	Mouse 4 1:400	Mouse 4 1:200	Mouse 4 1:100	IgG 1:800	IgG 1:800				
E	Mouse 5 1:3200	Mouse 5 1:1600	Mouse 5 1:800	Mouse 5 1:400	Mouse 5 1:200	Mouse 5 1:100	IgG 1:1600	IgG 1:1600				
F	Mouse 6 1:3200	Mouse 6 1:1600	Mouse 6 1:800	Mouse 6 1:400	Mouse 6 1:200	Mouse 6 1:100	IgG 1:3200	IgG 1:3200				
G	Mouse 7 1:3200	Mouse 7 1:1600	Mouse 7 1:800	Mouse 7 1:400	Mouse 7 1:200	Mouse 7 1:100	IgG 1:6400	IgG 1:6400				
H	Mouse 8 1:3200	Mouse 8 1:1600	Mouse 8 1:800	Mouse 8 1:400	Mouse 8 1:200	Mouse 8 1:100	IgG 1: 12,800	IgG 1: 12,800				
	Mouse 9 1:3200	Mouse 9 1:1600	Mouse 9 1:800	Mouse 9 1:400	Mouse 9 1:200	Mouse 9 1:100	IgG 1: 25,600	IgG 1: 25,600				
	Mouse 10	Mouse 10	Mouse 10 1:800	Mouse 10 1:400	Mouse 10 1:200	Mouse 10 1:100	Mouse 1 IgG 1: 51,200	IgG 1: 51,200				
	Mouse 11	Mouse 11	Mouse 11 1:800	Mouse 11 1:400	Mouse 11 1:200	Mouse 11 1:100	Blank	Blank				
	Mouse 12	Mouse 12	Mouse 12 1:800	Mouse 12 1:400	Mouse 12 1:200	Mouse 12 1:100	Blank	Blank				

[090] Serum ELISA: Serum anti-PA IgG was measured by ELISA. As shown in Table 2, two rows of a 96-well plate were coated with two-fold serial dilutions of mouse IgG (Sigma I-5381), beginning at 1 µg/ml in PBS, and continuing to 2 ng/ml in column 10, to serve as standards. Each row served as a replicate. The remaining four wells in the two rows served as blanks. Remaining wells of the plate were coated with 1 µg/ml PA in PBS pH7.2. Coating continued overnight at 4°C under high humidity. Wells were washed three times with PBS/0.05% Tween 20 (PBST), blocked 1 hour at room temperature with PBST + 3% FBS. Serum samples were serially diluted two-fold in PBST/FBS from 1:100 to 1:3,200. Serum dilutions were incubated on plates at 4°C overnight in high humidity. After again washing wells three times with PBST, mouse antibodies binding to PA were detected via 1:1000 dilution of HRP-conjugate goat anti-mouse IgG (Southern Biotech, #1030.05, Lot #D240-N742G) diluted into PBST/FBS, and incubated on wells for 1.5 hour at room temperature. Wells were washed three times with PBST, and the plate developed with ABTS (Pierce Cat # 37615) for 30 minutes at room temperature. OD_{405} readings were taken and values from the standards were plotted into a standard curve using SoftMax Pro software. Sample

absorbencies were used to interpolate antibody concentration from the standard curves. Data was exported to Excel (Microsoft®) for further analysis. If the CV (coefficient of variation, or 100*Standard Deviation/Mean) for the replicates was greater than 25, another replicate was performed. The exception to this rule was where the mean results were less than 10 µg/ml, in which case a CV of 150 was tolerated. Sample results reading above the standard curve range were deemed out of range, and were reanalyzed using a starting dilution of 1:1000. Sera from 12 mice were run on each plate (one per column), and each sample was replicated on a separate plate. At least 2 analyses were performed on each sample.

[091] Serum IgG: Serum drawn prior to the initial vaccination showed no measurable recognition of PA (data not shown). Serum anti-PA IgG responses were also measured at 4 and 8 weeks (Fig. 1). The 4 week samples reflected responses of animals 2 weeks following the first boost. At that time point, one mouse (out of five) immunized with PA5/IPX50 mounted a measurable IgG response of 10 µg/ml, however, none of the mice receiving PA5/MPL responded positively. Four of five mice receiving PA20/IPX50 also had measurable antigen-specific IgG levels (5-40 µg/ml, mean of 13 µg/ml for all five mice). Three of five mice receiving PA20/MPL responded (9-15 µg/ml, mean of 7 µg/ml for the group of five mice). The two vaccines containing 20 µg PA were not statistically different ($p<.44$).

[092] At 8 weeks, the mice immunized with PA20/IPX responded quite variably (29, 92, 96, 216 and 321 µg/ml), but all had positive values (treatment mean 150 µg/ml). Similar results were seen with the PA20/MPL immunized mice, in that responses were variable (19, 82, 104,162 and 221 µg/ml), and the treatment mean (118 µg/ml), although numerically lower, was statistically similar to that of PA20/IPX ($p<.61$). All mice immunized with PA5/IPX had postive responses at 8 weeks (15, 18, 18, 30 and 174 µg/ml, mean for treatment group (51 µg/ml), while none of the mice immunized with PA5/MPL had measurable anti-PA IgG levels. However, variability in responses to the PA5/IPX immunization was too great to show a significant difference from the PA5/MPL group ($p<.17$).

[093] LeTx challenge: All mice were rested for one week after the final sampling at eight weeks. One week later on 2/5/03, each mouse received approximately 6 LD_{50} of LeTx. TTD is shown in Table 3 and presented graphically in Fig. 2, with the exception that one mouse immunized with MPL only was excluded, as the LeTx entered subcutaneously rather than intravenously. Mice were either completely protected, or not at all, as TTD was not significantly extended in non-surviving mice with positive levels of anti-PA IgG over the TTDs of control mice. All mice receiving immunizations containing 20 µg PA survived, whether the immunization was adjuvanted by IPX or MPL, indicating that either of this vaccine formulations are sufficiently protective against LeTx challenge.

Table 3
LeTx Challenge

Group	Vaccine	TTD, hours	Median TTD, hours	Percent Surviving
1	MPL	33, S*, 29	37	33
2	IPX	45,26,27	27	0
3	PA5/IPX	20, 20, 33, S, S	29	40
4	PA20/IPX	S, S, S, S, S	undefined	100
5	PA5/MPL	24, 45, S, 45, 30	45	20
6	PA20/MPL	S, S, S, S, S	undefined	100
7	Naïve	27, 44, 30	30	0

TTD=Time To Death
*Received challenge dose subcutaneously rather than intravenously.
S=Survived >168 h (7 days)

[094] Mouse survival did not strictly correlate with level of serum IgG recognizing PA, as a mouse immunized with PA20/MPL survived with 18 µg/ml IgG, while two mice immunized with PA5/IPX50 with the same level of IgG did not survive. It is well known in the literature that protection from anthrax does not always correlate with titer. Although no LeTx administration difficulties were noted with the low-level responding PA20/MPL, it cannot be excluded that some of the toxin did not make it into the circulatory system.

[095] This study tested IPX50 and MPL as mucosal adjuvants for intranasal (IN) immunization with PA. Intranasal immunization of mice with 20 µg PA adjuvanted either with IPX50 or MPL protected mice from an intravenous LeTx challenge.

Example 2

Intranasal Vaccination with Protective Antigen

[096] Protocol: Female Balb/c mice, 17 weeks old, were immunized on days 0, 13, and 27 with 10 µl total volume of vaccine per animal. Prior to immunization, mice were lightly anesthetized with isofluorane to effect (2-4%), and 5 µl/nostril bolus was delivered to each nares. Retention of vaccine within the nares was generally good but when sneezing occurred it was noted.

[097] Vaccine Formulation: Mice were immunized with either 5 µg, 10 µg, or 20 µg PA. Lyophilized PA was obtained from List Biochemical (Cat. #171B, Lot #1714BA) and reconstituted in water immediately prior to use, which brought the salt content to 5 mM HEPES/50 mM NaCl. Invaplex 50 was stored at –80°C until immediately prior to use, at which time it was thawed on ice. MPL+TDM (MPL) (Sigma #M-6536, Lot #072K1313) was warmed to 40°C and resuspended in 1 mL 150 mM NaCl immediately prior to use.

Invaplex 50 (Lot #GNGO) was obtained from Edwin V. Oaks (Walter Reed Army Institute of Research (WRAIR)) Invaplex 50 was stored at –80°C. Immediately prior to use, Invaplex 50 was thawed on ice. Adjuvants were added to PA at 5 µl/dose immediately prior to use.

[098] Adjuvants: Invaplex 50 is an ion-exchange chromatography fraction of a water extract isolated from Shigella bacteria containing the Shigella invasion complex (U.S. Patent Nos. 6,680,374, 6,277,379, 6,245,892. MPL+TDM is monophosphoryl lipid A and synthetic trehalose dicorynomycolate in squalene and Tween 80.

[099] Treatment Groups: See Table 4.

Table 4
Treatment Groups

Group #	Treatment	Short name	No. mice
1	MPL+TDM	MPL	3
2	Invaplex 50	IPX	3
3	5 µg PA/Invaplex 50	PA5/IPX	5
4	10 µg PA/Invaplex 50	PA10/IPX	5
5	20 µg PA/Invaplex 50	PA20/IPX	5
6	5 µg PA/MPL+TDM	PA5/MPL	5
7	10 µg PA/MPL+TDM	PA10/MPL	5
8	20 µg PA/MPL+TDM	PA20/MPL	5
9	Naïve	Naïve	3

[0100] Sampling: Fecal and blood samples were collected from the mice prior to the initial vaccination (day 0) and on days 27 and 56 (prior to boosts).

[0101] Anthrax Lethal Toxin Challenge: One week following collection of final samples, mice received an intravenous challenge with approximately 6 LD_{50} of *B. anthracis* Lethal Toxin (LeTx). PA (60 µg) and LF (30 µg) (List Biochemical; PA #171B Lot #1712B; LF #172B Lot #1721B) were injected via tail vein in a total volume of 100 µl PBS. Mice were observed every 15 minutes for 16 hours post-injection, and hourly for hours 20 through 36. Thereafter, mice were checked every 6-8 hours for 7 days (168 hours). Time to death was recorded for each mouse that died. Mice that were still alive at 168 hours were defined as survivors.

[0102] Serum ELISA: Serum anti-PA IgG was measured by ELISA. Plate layout is shown in Table 5. The Softmax Pro template was originally designed by Susan Wimer-Mackin and updated by Sarah J Warwood. Two rows of a 96-well plate were coated with two-fold serial dilutions of mouse IgG (Sigma I-5381), beginning at 1 µg/ml in PBS, and continuing to 2 ng/ml in column 10, to serve as standards. Each row served as a replicate. The remaining four wells in the two rows served as blanks. Remaining wells of the plate

were coated with 1 µg/ml PA in phosphate buffered saline (PBS) pH7.2). Coating continued overnight at 4°C under high humidity. Wells were washed three times with PBS/0.05% Tween 20 (PBST), blocked for 1 hour at room temperature with PBST + 3% fetal bovine sera (FBS). Serum samples were serially diluted two-fold in PBST/FBS beginning at 1:100 to 1:3,200. Serum dilutions were incubated on plates at 4°C overnight in high humidity. After washing wells three times with PBST, mouse antibodies binding to PA were detected via 1:1000 dilution of HRP-conjugate goat anti-mouse IgG (Southern Biotech, #1030.05, Lot #D240-N742G) diluted into PBST/FBS, and incubated on wells for 1.5 hours at room temperature. Wells were again washed three times with PBST, and the plate developed with ABTS (Pierce Cat #37615) for 30 minutes at room temperature. OD_{405} readings were taken and values from the standards plotted into a standard curve using SoftMax Pro software. Sample absorbencies were used to interpolate antibody concentration from the standard curves. Data was exported to Excel (Microsoft®) for further analysis. If the CV (coefficient of variation, or 100*Standard Deviation/Mean) for the replicates was greater than 25, another replicate was performed. The exception to this rule was where the mean results were less than 10 µg/ml, in which case a CV of 150 was tolerated. Sample results reading above the standard curve range were deemed out of range, and were reanalyzed using a starting dilution of 1:1000.Sera from 12 mice were run on each plate (one per column), and each sample was replicated on a separate plate. At least 2 analyses were performed on each sample.

Table 5
ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Mouse 1:32001	Mouse 1:16001	Mouse 1:800	Mouse 1:400	Mouse 1:200	Mouse 1:100	Mouse 1:6400	Mouse 1:3200	Mouse 1:1600	Mouse 1:800	Mouse 1:400	Mouse 1:200
B	Mouse 2:32002	Mouse 1:16002	Mouse 1:800	Mouse 2:400	Mouse 1:200	Mouse 1:100	Mouse 2:6400	Mouse 2:3200	Mouse 2:1600	Mouse 2:800	Mouse 2:400	Mouse 2:200
C	Mouse 3:32003	Mouse 1:16003	Mouse 1:800	Mouse 3:400	Mouse 1:200	Mouse 1:100	Mouse 3:6400	Mouse 3:3200	Mouse 3:1600	Mouse 3:800	Mouse 3:400	Mouse 3:200
D	Mouse 4:32004	Mouse 1:16004	Mouse 1:800	Mouse 4:400	Mouse 1:200	Mouse 1:100	Mouse 4:6400	Mouse 4:3200	Mouse 4:1600	Mouse 4:800	Mouse 4:400	Mouse 4:200
E	Mouse 5:32005	Mouse 1:16005	Mouse 1:800	Mouse 5:400	Mouse 5:200	Mouse 5:100	Mouse 5:6400	Mouse 5:3200	Mouse 5:1600	Mouse 5:800	Mouse 5:400	Mouse 5:200
F	Mouse 6:32006	Mouse 1:16006	Mouse 1:800	Mouse 6:400	Mouse 6:200	Mouse 6:100	Mouse 6:6400	Mouse 6:3200	Mouse 6:1600	Mouse 6:800	Mouse 6:400	Mouse 6:200
G	Mouse 7:32007	Mouse 1:16007	Mouse 1:800	Mouse 7:400	Mouse 7:200	Mouse 7:100	Mouse 7:6400	Mouse 7:3200	Mouse 7:1600	Mouse 7:800	Mouse 7:400	Mouse 7:200
H	Mouse 8:32008	Mouse 1:16008	Mouse 1:800	Mouse 8:400	Mouse 8:200	Mouse 8:100	Mouse 8:6400	Mouse 8:3200	Mouse 8:1600	Mouse 8:800	Mouse 8:400	Mouse 8:200
	Mouse 9:32009	Mouse 1:16009	Mouse 1:800	Mouse 9:400	Mouse 9:200	Mouse 9:100	Mouse 9:6400	Mouse 9:3200	Mouse 9:1600	Mouse 9:800	Mouse 9:400	Mouse 9:200
	Mouse 10	Mouse 10	Mouse 10	Mouse 10	Mouse 10	Mouse 10	Mouse 10	Mouse 10	Mouse 10	Mouse 10	Mouse 10	Mouse 10
	Mouse 11	Mouse 11	Mouse 11	Mouse 11	Mouse 11	Mouse 11	Mouse 11	Mouse 11	Mouse 11	Mouse 11	Mouse 11	Mouse 11
	Mouse 12	Mouse 12	Mouse 12	Mouse 12	Mouse 12	Mouse 12	Mouse 12	Mouse 12	Mouse 12	Mouse 12	Mouse 12	Mouse 12

	1	2	3	4	5	6	7	8	9	10	11	12
A	Mouse1 1:100	Mouse1 1:100	Mouse1 1:100	Mouse1 1:100	Mouse1 1:100	Mouse1 1:100	Mouse1 1:100	Mouse1 1:100	Mouse1 1:100	Mouse1 1:100	Mouse1 1:100	Mouse1 1:100
B	IgG 1:100	IgG 1:200	IgG 1:400	IgG 1:800	IgG 1:1600	IgG 1:3200	IgG 1:6400	IgG 1: 12.800	IgG 1: 25.600	IgG 1: 51.200	Blank	Blank
C	Mouse2 1:100	Mouse2 1:100	Mouse2 1:100	Mouse2 1:100	Mouse2 1:100	Mouse2 1:100	Mouse2 1:100	Mouse2 1:100	Mouse2 1:100	Mouse2 1:100	Mouse2 1:100	Mouse2 1:100
D	Mouse2 1:200	Mouse2 1:200	Mouse2 1:200	Mouse2 1:200	Mouse2 1:200	Mouse2 1:200	Mouse2 1:200	Mouse2 1:200	Mouse2 1:200	Mouse2 1:200	Mouse2 1:200	Mouse2 1:200
E	Mouse3 1:100	Mouse3 1:100	Mouse3 1:100	Mouse3 1:100	Mouse3 1:100	Mouse3 1:100	Mouse3 1:100	Mouse3 1:100	Mouse3 1:100	Mouse3 1:100	Mouse3 1:100	Mouse3 1:100
F	Mouse3 1:200	Mouse3 1:200	Mouse3 1:200	Mouse3 1:200	Mouse3 1:200	Mouse3 1:200	Mouse3 1:200	Mouse3 1:200	Mouse3 1:200	Mouse3 1:200	Mouse3 1:200	Mouse3 1:200
G	Mouse4 1:100	Mouse4 1:100	Mouse4 1:100	Mouse4 1:100	Mouse4 1:100	Mouse4 1:100	Mouse4 1:100	Mouse4 1:100	Mouse4 1:100	Mouse4 1:100	Mouse4 1:100	Mouse4 1:100
H	Mouse4 1:200	Mouse4 1:200	Mouse4 1:200	Mouse4 1:200	Mouse4 1:200	Mouse4 1:200	Mouse4 1:200	Mouse4 1:200	Mouse4 1:200	Mouse4 1:200	Mouse4 1:200	Mouse4 1:200
	Mouse5 1:100	Mouse5 1:100	Mouse5 1:100	Mouse5 1:100	Mouse5 1:100	Mouse5 1:100	Mouse5 1:100	Mouse5 1:100	Mouse5 1:100	Mouse5 1:100	Mouse5 1:100	Mouse5 1:100
	Mouse5 1:200	Mouse5 1:200	Mouse5 1:200	Mouse5 1:200	Mouse5 1:200	Mouse5 1:200	Mouse5 1:200	Mouse5 1:200	Mouse5 1:200	Mouse5 1:200	Mouse5 1:200	Mouse5 1:200
	Mouse6 1:100	Mouse6 1:100	Mouse6 1:100	Mouse6 1:100	Mouse6 1:100	Mouse6 1:100	Mouse6 1:100	Mouse6 1:100	Mouse6 1:100	Mouse6 1:100	Mouse6 1:100	Mouse6 1:100
	Mouse6 1:200	Mouse6 1:200	Mouse6 1:200	Mouse6 1:200	Mouse6 1:200	Mouse6 1:200	Mouse6 1:200	Mouse6 1:200	Mouse6 1:200	Mouse6 1:200	Mouse6 1:200	Mouse6 1:200
	Mouse7 1:100	Mouse7 1:100	Mouse7 1:100	Mouse7 1:100	Mouse7 1:100	Mouse7 1:100	Mouse7 1:100	Mouse7 1:100	Mouse7 1:100	Mouse7 1:100	Mouse7 1:100	Mouse7 1:100
	Mouse7 1:200	Mouse7 1:200	Mouse7 1:200	Mouse7 1:200	Mouse7 1:200	Mouse7 1:200	Mouse7 1:200	Mouse7 1:200	Mouse7 1:200	Mouse7 1:200	Mouse7 1:200	Mouse7 1:200
	Mouse8 1:100	Mouse8 1:100	Mouse8 1:100	Mouse8 1:100	Mouse8 1:100	Mouse8 1:100	Mouse8 1:100	Mouse8 1:100	Mouse8 1:100	Mouse8 1:100	Mouse8 1:100	Mouse8 1:100
	Mouse8 1:200	Mouse8 1:200	Mouse8 1:200	Mouse8 1:200	Mouse8 1:200	Mouse8 1:200	Mouse8 1:200	Mouse8 1:200	Mouse8 1:200	Mouse8 1:200	Mouse8 1:200	Mouse8 1:200
	Mouse9 1:100	Mouse9 1:100	Mouse9 1:100	Mouse9 1:100	Mouse9 1:100	Mouse9 1:100	Mouse9 1:100	Mouse9 1:100	Mouse9 1:100	Mouse9 1:100	Mouse9 1:100	Mouse9 1:100
	Mouse9 1:200	Mouse9 1:200	Mouse9 1:200	Mouse9 1:200	Mouse9 1:200	Mouse9 1:200	Mouse9 1:200	Mouse9 1:200	Mouse9 1:200	Mouse9 1:200	Mouse9 1:200	Mouse9 1:200
	Mouse10 1:100	Mouse10 1:100	Mouse10 1:100	Mouse10 1:100	Mouse10 1:100	Mouse10 1:100	Mouse10 1:100	Mouse10 1:100	Mouse10 1:100	Mouse10 1:100	Mouse10 1:100	Mouse10 1:100
	Mouse10 1:200	Mouse10 1:200	Mouse10 1:200	Mouse10 1:200	Mouse10 1:200	Mouse10 1:200	Mouse10 1:200	Mouse10 1:200	Mouse10 1:200	Mouse10 1:200	Mouse10 1:200	Mouse10 1:200
	Mouse11 1:100	Mouse11 1:100	Mouse11 1:100	Mouse11 1:100	Mouse11 1:100	Mouse11 1:100	Mouse11 1:100	Mouse11 1:100	Mouse11 1:100	Mouse11 1:100	Mouse1 Blank	Blank
	Mouse11 1:200	Mouse11 1:200	Mouse11 1:200	Mouse11 1:200	Mouse11 1:200	Mouse11 1:200	Mouse11 1:200	Mouse11 1:200	Mouse11 1:200	Mouse11 1:200		
	Mouse12 1:100	Mouse12 1:100	Mouse12 1:100	Mouse12 1:100	Mouse12 1:100	Mouse12 1:100	Mouse12 1:100	Mouse12 1:100	Mouse12 1:100	Mouse12 1:100		
	Mouse12 1:200	Mouse12 1:200	Mouse12 1:200	Mouse12 1:200	Mouse12 1:200	Mouse12 1:200	Mouse12 1:200	Mouse12 1:200	Mouse12 1:200	Mouse12 1:200		

[0103] Serum IgG: Serum drawn prior to the initial vaccination showed no measurable recognition of PA (data not shown). Serum anti-PA IgG responses were also measured at 4 and 8 weeks (Fig. 3). The 4 week samples reflected responses of animals 2 weeks following the first boost. At that time point, four out of five mice immunized with PA5/IPX mounted measurable IgG responses (range of 3-12 μg/ml, mean for entire group 5 μg/ml). All five mice immunized with PA10/IPX had measurable responses, with one mouse recording a very significant 82 μg/ml (range of 9-82 μg/ml, mean of 28 μg/ml). Four of five mice receiving PA20/IPX also had measurable antigen-specific IgG levels (range of 9-22 μg/ml, mean of 12 μg/ml for all five mice).

[0104] Mice immunized with PA20/IPX responded at 8 weeks (27, 204, 32, 62 and 143 μg/ml; treatment mean 93 μg/ml). Results with PA10/IPX immunized mice (145, 315, 117, 89 and 50 μg/ml) (treatment mean 143 μg/ml) were statistically similar to PA20/IPX immunized mice ($p<.41$). All mice immunized with PA5/IPX had positive responses at 8 weeks (61, 60, 33, 59 and 4 μg/ml, mean for treatment group (43 μg/ml). Two mice immunized with PA + MPL mounted measurable anti-PA IgG responses. One of those mice

was immunized with PA10/MPL had 17 μg/ml anti-PA IgG, and the other was immunized with PA20/MPL had 3 μg/ml.

[0105] LeTx challenge: All mice were rested for one week after the final sampling at eight weeks. Each mouse received approximately 6 LD_{50} LeTx. Time to death (TTD) is shown in Table 6 and presented graphically in Fig. 4. Mice were either completely protected, or not at all, as TTD was not significantly extended in non-surviving mice with positive levels of anti-PA IgG over the TTDs of control mice. All mice receiving PA10/IPX50, four out of the five mice receiving PA20/IPX50 and three of five mice receiving PA5/IPX50 survived the challenge. The PA20/IPX50 mouse that died had relatively low anti-PA IgG (32 μg/ml), as did one of the PA5/IPX50 mice that died (4 μg/ml). The other PA5/IPX50 mouse that died had 60 μg/ml anti-PA IgG idea that anti-PA titers do not necessarily correlate with toxin neutralization titers. Only one animal receiving PA adjuvanted with MPL survived, and we questioned at the time of challenge if all of that mouse's LeTx was given intravenously. This mouse had a barely measurable anti-PA IgG response in its serum (<1 μg/ml), suggesting that the challenge was indeed incompletely given.

Table 6
LeTx Challenge

Group	Treatment	TTD* (hr)	Median TTD (hr)	Number Surviving
1	MPL	27, 33, 25	27	0/3
2	IPX50	33, 33, 26	33	0/3
3	PA5/IPX50	**S**, 33, **S**, **S**, 24	undefined	3/5
4	PA10/IPX50	**S, S, S, S, S,**	undefined	5/5
5	PA20/IPX50	**S, S,** 35, **S, S,**	undefined	4/5
6	PA5/MPL	29, 33, 27, 33, **S***	33	1/5
7	PA10/MPL	30, 18, 33, 34, 22	30	0/5
8	PA20/MPL	35, 29, 57, 36, 26	35	0/5
9	Naïve	35, 24, 71	35	0/3

TTD=Time to death
*Received at last past of challenge dose subcutaneously rather than intravenously
S=Survived >168 hrs (7 days) after 6 LD_{50} LeTx i.v.

[0106] Mouse survival did not strictly correlate with level of serum IgG recognizing PA. It is well known in the literature that protection from anthrax does not always correlate with titer.

[0107] Mean serum anti-PA IgG responses for Example 1 and Example 2 are shown in Table 7. No statistical differences between the two studies were detected for the PA/IPX50 treatments.

Table 7
Mean anti-PA IgG Examples 1 & 2

	4 weeks		8 weeks	
Study	Ex. 1	Ex. 2	Ex. 1	Ex. 2
MPL	0	0	0	0
IPX	0.0	0.0	0.0	0.0
PA5/IPX50	2.0	5.2	51.0	43.2
PA10/IPX50	-	27.7	-	143.3
PA20/IPX50	13.4	12.2	150.7	93.6
PA5/MPL	0.0	0.0	0.0	0.1
PA10/MPL	-	0.0	-	3.4
PA20/MPL	7.2	0.0	117.5	0.6
Naïve	0.0	0.0	0.0	0.0

Example 3

Intranasal and Intraperitoneal Vaccination with Protective Antigen

[0108] Protocol: Female C57Bl mice, 16 weeks old, were immunize on Days 0 and 21 with 23 µg PA. Intraperitoneal (IP) immunizations were in a total volume of 100 µl containing 5 µl MPL per the manufacturer's directions. IN immunizations were in a total volume of 10 µl, and each contained 5 µg IPX50. For IN immunizations, a 5 µl/nostril bolus was delivered to each nares after lightly anesthetizing the mouse via isofluorine to effect.

[0109] Nanoparticles: Nanoparticles (NPs) were 20% nickel chelate, 32% EAPDA, 48% PCDA Na. PA in the form of a histidine tagged (His-tagged) recombinant protein was purified via IMAC (Batch 4, 5/17/02). His-tagged PA was used in vaccines where the PA was bound to NPs (designated PA*NP) and in PA vaccines that did not contain NPs. All other PA containing vaccines utilized native, non-His-tagged PA.

[0110] Vaccine formulation: His-tagged PA was bound to NPs with gentle mixing at 4°C overnight immediately prior to immunization. Non-conjugated NPs were treated similarily, only PA was not His-tagged, and thus was not suitable to bind NPs. Adjuvants were added to vaccines at 5 µl/dose immediately prior to use. PA-containing vaccines had 23 µg/dose. Parenteral vaccines were diluted up to 100 µl each with 150 mM NaCl. MPL (Sigma #M-6536, Lot #42K1185) was warmed to 40°C and resuspended in 1 mL 150 mM NaCl just prior to use. IPX50 (lot #GNGO) was obtained from Edwin V. Oaks (WRAIR). IPX50 was stored at –80°C until immediately prior to use and thawed on ice. Adjuvants were added to PA at 5 µl/dose immediately prior to use.

[0111] Adjuvants: IPX50 is an ion-exchange chromatography fraction of a water extract isolated from Shigella bacteria containing the Shigella invasion complex. MPL is composed

of monophosphoryl lipid A and synthetic treholose dicorynomycolate in squalene and Tween 80.

[0112] Treatment groups: Treatment groups are shown in Table 8. Negative controls were Naïve mice, and mice receiving either nanoparticles and MPL (IP), nanoparticles and Invaplex (IN) or Invaplex (IN) alone without the addition of PA. PA/MPL was included as a positive control.

Table 8
Treatment Groups

Group #	Treatment	Short name	Route	No. mice
1	NP/MPL	NP/MPL	IP	3
2	NP/Invaplex 50	NP/IPX	IN	3
3	PA/MPL	PA/MPL	IP	5
4	PA/Invaplex 50	PA/IPX	IN	5
5	PA bound NP/MPL	PA*NP/MPL	IP	5
6	PA bound NP/Invaplex 50	PA*NP/IPX	IN	5
7	Naïve	Naïve	-	3
8	PA unbound NP/MPL	PA/NP/MPL	IP	5
9	PA unbound NP/Invaplex 50	PA/NP/IPX	IN	5
10	Invaplex only	IPX	IN	3

[0113] Sampling: Fecal and blood samples were collected from the mice prior to the initial vaccination (Day 0) and on Days 21 and 56. Mice were boosted with a repeat of the initial vaccination after the Day 21 samples were collected. Animals were sacrificed at the end of this study and the serum was collected and stored.

[0114] Serum ELISA: Serum anti-PA IgG was measured by ELISA (Table 9). Two rows of a 96-well plate were coated with two-fold serial dilutions of mouse IgG (Sigma I-5381) from 1 µg/ml in PBS to 2 ng/ml in column 10 to serve as standards. Each row served as a replicate. The remaining four wells in the two rows served as blanks. Remaining wells of the plate were coated with 1 µg/ml PA in PBS pH7.2. Coating continued overnight at 4°C under high humidity. Wells were washed three times with PBS/0.05% Tween 20 (PBST), blocked 1 hour at room temperature with PBST + 3% FBS. Serum samples were serially diluted two-fold in PBST/FBS from 1:100 to 1:3,200. Serum dilutions were incubated on plates at 4°C overnight in high humidity. After again washing wells three times with PBST, mouse antibodies binding to PA were detected via 1:1000 dilution of HRP-conjugate goat anti-mouse IgG (Southern Biotech #1030.05, Lot #D240-N742G) diluted into PBST/FBS, and incubated on wells for 1.5 hours at room temperature. Wells were washed three times with PBST and the plate developed with ABTS (Pierce Cat # 37615) for 30 minutes at room

temperature. OD_{405} readings were taken and values from the standards plotted into a standard curve using SoftMax Pro software. Sample absorbencies were used to interpolate antibody concentration from the standard curves. Data was exported to Excel for further analysis. Sera from 12 mice were run on each plate (one per column), and each sample was replicated on a separate plate. At least 2 analyses were performed on each sample.

Table 9
ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	Mouse1 1:3200	Mouse2 1:3200	Mouse3 1:3200	Mouse4 1:3200	Mouse 5 1:3200	Mouse6 1:3200	Mouse7 1:3200	Mouse8 1:3200	Mouse9 1:3200	Mouse 10 1:800	Mouse 11	Mouse 12
D	Mouse1 1:200	Mouse2 1:200	Mouse3 1:200	Mouse4 1:200	Mouse 5 1:200	Mouse 6 1:200	Mouse 7 1:200	Mouse 8 1:200	Mouse 9 1:200	Mouse 10 1:400	Mouse 11 1:400	Mouse 12 1:400
E	Mouse 1 1:400	Mouse 2 1:400	Mouse 3 1:400	Mouse 4 1:400	Mouse 5 1:400	Mouse 6 1:400	Mouse 7 1:400	Mouse 8 1:400	Mouse 9 1:400	Mouse 10 1:400	Mouse 11 1:400	Mouse 12 1:400
F	Mouse 1 1:800	Mouse 2 1:800	Mouse 3 1:800	Mouse 4 1:800	Mouse 5 1:800	Mouse 6 1:800	Mouse 7 1:800	Mouse 8 1:800	Mouse 9 1:800	Mouse 10 1:800	Mouse 11 1:800	Mouse 12 1:800
G	Mouse 1 1:1600	Mouse 2 1:1600	Mouse 3 1:1600	Mouse 4 1:1600	Mouse 5 1:1600	Mouse 6 1:1600	Mouse 7 1:1600	Mouse 8 1:1600	Mouse 9 1:1600	Mouse 10 1:1600	Mouse 11 1:1600	Mouse 12 1:1600
H	Mouse 1 1:3200	Mouse 2 1:3200	Mouse 3 1:3200	Mouse 4 1:3200	Mouse 5 1:3200	Mouse 6 1:3200	Mouse 7 1:3200	Mouse 8 1:3200	Mouse 9 1:3200	Mouse 10	Mouse 11	Mouse 12

	1	2	3	4	5	6	7	8	9	10	11	12
A	IgG 1:100	IgG 1:200	IgG 1:400	IgG 1:800	IgG 1:1600	IgG 1:3200	IgG 1:6400	IgG 1: 12,800	IgG 1: 25,600	IgG 1: 51,200	Blank	Blank
B	IgG 1:100	IgG 1:200	IgG 1:400	IgG 1:800	IgG 1:1600	IgG 1:3200	IgG 1:6400	IgG 1: 12,800	IgG 1: 25,600	IgG 1: 51,200	Blank	Blank
C	Mouse1 1:100	Mouse2 1:100	Mouse3 1:100	Mouse4 1:100	Mouse 5 1:100	Mouse6 1:100	Mouse7 1:100	Mouse8 1:100	Mouse9 1:100	Mouse 10 1:100	Mouse1 1:200	
D	Mouse1 1:200	Mouse2 1:200	Mouse3 1:200	Mouse4 1:200	Mouse 5 1:200	Mouse 6 1:200	Mouse 7 1:200	Mouse 8 1:200	Mouse 9 1:200	Mouse 10 1:200	Mouse 11 1:200	
E	Mouse 1 1:400	Mouse 2 1:400	Mouse 3 1:400	Mouse 4 1:400	Mouse 5 1:400	Mouse 6 1:400	Mouse 7 1:400	Mouse 8 1:400	Mouse 9 1:400	Mouse 10 1:400	Mouse 11 1:400	
F	Mouse 1 1:800	Mouse 2 1:800	Mouse 3 1:800	Mouse 4 1:800	Mouse 5 1:800	Mouse 6 1:800	Mouse 7 1:800	Mouse 8 1:800	Mouse 9 1:800	Mouse 10 1:800	Mouse 11 1:800	
G	Mouse 1 1:1600	Mouse 2 1:1600	Mouse 3 1:1600	Mouse 4 1:1600	Mouse 5 1:1600	Mouse 6 1:1600	Mouse 7 1:1600	Mouse 8 1:1600	Mouse 9 1:1600	Mouse 10	Mouse 11	
H	Mouse 1 1:3200	Mouse 2 1:3200	Mouse 3 1:3200	Mouse 4 1:3200	Mouse 5 1:3200	Mouse 6 1:3200	Mouse 7 1:3200	Mouse 8 1:3200	Mouse 9 1:3200	Mouse 10	Mouse 11	Mouse 12

[0115] Antibody responses: Serum drawn prior to the initial vaccination showed no measurable recognition of PA (data not shown). Serum anti-PA responses were measured in mice three weeks post immunization, immediately prior to the boost. Mice receiving PA/MPL (delivered IP, no NP) had the greatest mean anti-PA responses in their sera at 31 µg/ml, with values ranging from 22 to 44 µg/ml. The mean response of PA/NP/MPL (free PA mixed with but not bound to NP, IP delivery) mice was statistically similar at 26 µg/ml (unpaired t-test, p>.05), with values ranging from 9-51 µg/ml. Four of the five mice receiving PA*NP/MPL (PA bound to NPs delivered IP) had measurable anti-PA IgG levels (range 0 to 14 µg/ml), but the group mean response was 6 µg/ml IgG, which was significantly lower than the other two PA-containing vaccines delivered IP (p<.05, Fig. 5). Two out of five mice

receiving PA/IPX intranasally responded to nasally delivered PA. None of the control mice (Naïve, NP/MPL, NP/IPX or IPX treatments) had measurable anti-PA responses, as expected.

[0116] Results from eight week serum samples (five weeks following the boost) are shown in FIG. 6. Mice receiving PA/MPL (no NP, IP delivery) had the greatest seral anti-PA IgG responses (range of 58-284 µg/ml, mean of 175 µg/ml). The increase in mean response by PA/MPL mice compared to PA/NP/MPL mice approached statistical significance ($p<.08$). PA/NP/MPL mice responded with values ranging from 8-172 µg/ml anti-PA IgG, with a treatment mean of 82 µg/ml. Mice receiving PA bound to NP via IP delivery (PA*NP/MPL) had responses lower than those of PA/MPL mice ($p<.05$), but similar to those receiving the same NPs, but with unbound PA ($p>.05$). Values for the PA*NP/MPL mice ranged from 3 µg/ml to 132 µg/ml with a mean value of 70 µg/ml.

[0117] At eight weeks, mice receiving PA/IPX intranasally had a mean seral anti-PA IgG response of 37 µg/ml (range of 4-49 µg/ml). This response was statistically similar to both PA/NP treatments, but less than PA/MPL ($p<.05$). One mouse receiving PA/NP/IPX had measurable sera response of 13 µg/ml, however none of the others in that treatment had positive responses.

[0118] Nanoparticle effects on immune responses: His-binding NP approach of delivering an antigen did not appear advantageous. In fact, the NP inhibited anti-PA serum IgG.

Example 4

Intranasal Immunization with Multiple Anthrax Peptides

[0119] Protocol: Female Balb/c mice, 8 weeks old, were immunized on Day 0, 14, and 28. Serum samples were collected on days 0, 28, and 56. IN immunizations were in a total volume of 10 µl, and each contained 5 µg IPX50. For IN immunizations, a 5 µl/nostril bolus was delivered to each nares after lightly anesthetizing the mouse via isofluorine to effect.

[0120] Vaccine formulation: Adjuvants were added to vaccines at 5 µl/dose immediately prior to use. PA was administered at 10 µg/dose; Capsular 10-mer at 10 µg/dose; Capsule PGA at 10 µg/dose. IPX50 was obtained from Edwin V. Oaks (WRAIR). IPX50 was stored at –80°C until immediately prior to use and thawed on ice. Adjuvants were added to PA at 5 µl/dose immediately prior to use. PA conjugates were made according to the method of Schneerson *et al.* (Proc Natl Acad Sci USA. 2003 Jul 22;100(15):8945-50. Epub 2003 Jul 11). Vaccines formulated with cholera toxin (CT) contained 1 microgram CT.

[0121] Adjuvants: IPX50 is an ion-exchange chromatography fraction of a water extract isolated from Shigella bacteria containing the Shigella invasion complex. CT is cholera toxin.

[0122] Treatment groups: Treatment groups are shown in Tables 10 and 11.

[0123] Sampling: Serum samples were collected on days 0, 28, and 56.

[0124] Serum ELISA: Serum anti-PA and anti-Capsule IgG were measured by ELISA and the amount of IgG to each antigen was interpolated from standard curves generated in parallel as described above.

[0125] Antibody responses: Serum drawn prior to the initial vaccination showed no measurable recognition of PA (data not shown).

[0126] Serum anti-PA responses were measured in mice 8 weeks post immunization (Table 10, Fig. 7). Mice receiving PA with or without Capsule or the capsule 10-mer with CT had mean anti-PA IgG responses ranging from 340-428 U/mL. Mice who received PA with IPX50 had measurable anti-PA IgG but the mean were responses ranged from 30-48 U/mL and some mice in each of these groups had no measurable response. The presence of the capsule or capsule 10-mer in these animals appeared to have no effect on the anti-PA response in mice receiving formulations with IPX50 or CT.

Table 10
Anti-PA IgG

Group	Treatment	Mean, U/mL	Standard Deviation
1	Naive	0.0	0.0
2	PA	0.0	0.0
3	IPX	0.0	0.0
4	PA+IPX	48.1	59.2
5	10mer+IPX	0.0	0.0
6	PA+Caps+IPX	30.7	13.5
7	PA conj 10mer+IPX	0.5	1.0
8	PA+10mer+IPX	47.4	44.0
9	PA+CT	340.6	114.2
10	PA+Caps+CT	353.4	142.3
11	PA+10mer+CT	428.1	163.0

[0127] In contrast, formulations with CT did not uniformly enhance the anti-capsule IgG response (Fig. 8). The greatest responses were observed in mice receiving PA + Caps + CT but the response within the group was variable. And the responses of mice receiving PA + 10-mer + CT on average were similar to mice receiving PA + CT. The most uniform and consistent anti-Capsule response in comparison to the other groups was observed in mice receiving PAconj10-mer + IPX50. The mean of 11.2 U/mL for this group exceeded the

PA + Caps + CT. Furthermore, all of the mice receive PAconj10-mer + IPX50 exceeded the responses observed in 4/5 mice in the PA + Caps + CT group.

Table 11

Anti-Capsule IgG

Group	Treatment	Mean, U/mL	Standard Deviation
1	Naive	1.1	0.3
2	PA	2.0	1.3
3	IPX	2.4	0.5
4	PA+IPX	2.8	1.9
5	10mer+IPX	2.0	0.9
6	PA+Caps+IPX	2.7	1.3
7	PA conj 10mer+IPX	11.2	1.7
8	PA+10mer+IPX	2.7	1.2
9	PA+CT	2.7	1.5
10	PA+Caps+CT	7.7	4.6
11	PA+10mer+CT	2.5	2.0

CLAIMS

What is claimed is:

1. A method of inducing an immune response to anthrax antigen comprising administering to a subject a composition comprising an anthrax peptide and a mucosal adjuvant.

2. The method according to claim 1, wherein said composition further comprises a second anthrax peptide.

3. The method according to claim 1, further comprising administering to said subject a second composition comprising a second anthrax peptide and a mucosal adjuvant.

4. A method of neutralizing an anthrax exotoxin in a subject comprising administering to said subject an immunogenic composition comprising:

i) an anthrax exotoxin peptide; and

ii) a mucosal adjuvant;

under conditions such that an immune response is induced in said subject and said exotoxin is neutralized.

5. A method of inducing immunity to an anthrax bacterium comprising administering to a subject an composition comprising an anthrax peptide and a mucosal adjuvant.

6. A method of protecting a subject from anthrax disease comprising vaccinating said subject with a composition comprising an anthrax peptide and a mucosal adjuvant.

7. The method according to claim 1, 2, 3, 4, 5, or 6, wherein said anthrax peptide is PA.

8. The method according to claim 1, 2, 3, 4, 5, or 6, wherein said anthrax peptide is LF.

9. The method according to claim 1, 2, 3, 4, 5, or 6, wherein said anthrax peptide is EF.

10. The method according to claim 1, 2, 3, 5, or 6, wherein said anthrax is PGA.

11. The method according to claim 1, 2, 3, 4, 5, or 6, wherein said mucosal adjuvant comprises a component of a gram-negative bacterial cell wall or outer layer.

12. The method according to claim 11, wherein said component is an invasin protein.

13. The method according to claim 12, wherein said invasin protein is an *E. coli* invasin protein.

14. The method according to claim 12, wherein said invasin protein is a Shigella invasin protein.

15. The method according to claim 13 or 14, wherein said mucosal adjuvant comprises Invaplex.

16. The method according to claim 15, wherein said Invaplex is Invaplex50.

17. The method according to claim 15, wherein said Invaplex is Invaplex25.

18. The method according to claim 11, wherein said component is lipid A.

19. The method according to claim 18, wherein said lipid A is monophosphoryl lipid A.

20. The method according to claim 15, wherein said mucosal adjuvant comprises TDM (synthetic trehalose dicorynomycolate).

21. The method according to claim 1, 2, 3, 4, 5, or 6, wherein the immune response is a T cell response.

22. The method according to claim 1, 2, 3, 4, 5, or 6, wherein the immune response is a B cell response.

23. The method according to claim 22, wherein said B cell response is an IgG or an IgA response.

24. The method according to claim 1, 2, 3, 4, 5, or 6, wherein the immune response is an anamnestic response.

25. The method according to claim 1, 2, 3, 4, 5, or 6, wherein the immune response protects said subject from anthrax disease.

26. The method according to claim 1, 2, 3, 4, 5, or 6, wherein said immune response ameliorates a symptom of anthrax disease.

27. The method according to claim 1, 2, 3, 4, 5, or 6, wherein said composition is administered to a mucosal surface of said subject.

28. The method according to claim 27, wherein said mucosal surface is a respiratory tract mucosal surface.

29. The method according to claim 28, wherein said respiratory tract mucosal surface as an intranasal mucosal surface.

30. A immunogenic composition comprising an anthrax peptide and a mucosal adjuvant.

31. The composition according to claim 30, wherein said anthrax peptide is PA.

32. The composition according to claim 30, wherein said anthrax peptide is LF.

33. The composition according to claim 30, wherein said anthrax peptide is EF.

34. The composition according to claim 30, wherein said anthrax is PGA.

35. The composition according to claim 30, wherein said composition further comprises a second anthrax peptide.

36. The composition according to claim 30, wherein said mucosal adjuvant comprises a component of a gram-negative bacterial cell wall or outer layer.

37. The composition according to claim 36, wherein said component is an invasin protein.

38. The composition according to claim 37, wherein said invasin protein is an E. coli invasin protein.

39. The composition according to claim 37, wherein said invasin protein is a Shigella invasin protein.

40. The composition according to claim 38 or 39, wherein said mucosal adjuvant comprises Invaplex.

41. The composition according to claim 40, wherein said Invaplex is Invaplex50.

42. The composition according to claim 40, wherein said Invaplex is Invaplex25.

43. The composition according to claim 30, wherein said component is lipid A.

44. The composition according to claim 43, wherein said lipid A is monophosphoryl lipid A.

45. The composition according to claim 44, wherein said mucosal adjuvant comprises TDM (synthetic trehalose dicorynomycolate).

46. The composition according to claim 30, wherein said composition is a vaccine suitable for inducing protective immunity to anthrax disease in a subject.

47. The composition according to claim 30, wherein said composition is suitable for treating anthrax disease in a subject.

48. The composition according to claim 40, wherein said composition suitable for administration to a mucosal surface of a subject.

49. The composition according to claim 48, wherein said mucosal surface is a respiratory tract mucosal surface.

ANTHRAX ANTIGENS AND METHODS OF USE

ABSTRACT OF THE DISCLOSURE

Anthrax antigens are provided that find use as immunogens and vaccines.

FIG. 1

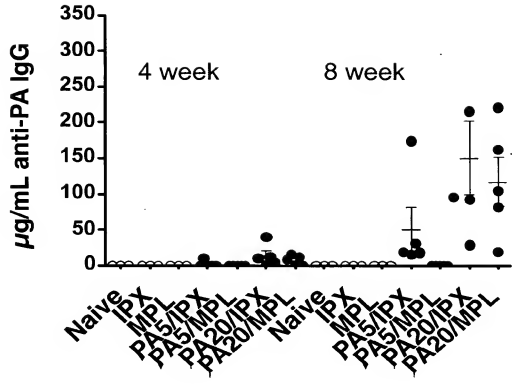

μg/mL anti-PA IgG
350
300
250
200
150
100
50
0
4 week
8 week
Naive
IPX
MPL
PA5/IPX
PA5/MPL
PA20/IPX
PA20/MPL
Naive
IPX
MPL
PA5/IPX
PA5/MPL
PA20/IPX
PA20/MPL

FIG. 2

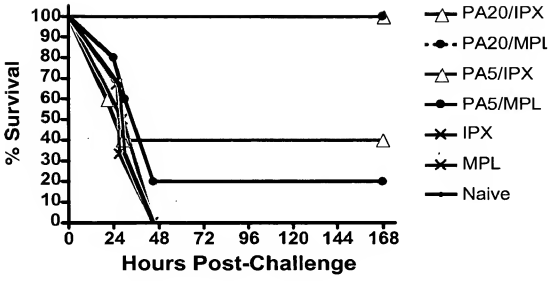

% Survival
100
90
80
70
60
50
40
30
20
10
0
0
24
48
72
96
120
144
168
Hours Post-Challenge
PA20/IPX
PA20/MPL
PA5/IPX
PA5/MPL
IPX
MPL
Naive

FIG. 3

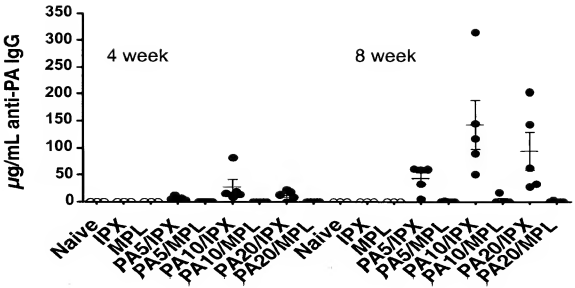

μg/mL anti-PA IgG
350
300
250
200
150
100
50
0
4 week
8 week
Naive
IPX
MPL
PA5/IPX
PA5/MPL
PA10/IPX
PA10/MPL
PA20/IPX
PA20/MPL
Naive
IPX
MPL
PA5/IPX
PA5/MPL
PA10/IPX
PA10/MPL
PA20/IPX
PA20/MPL

FIG. 4

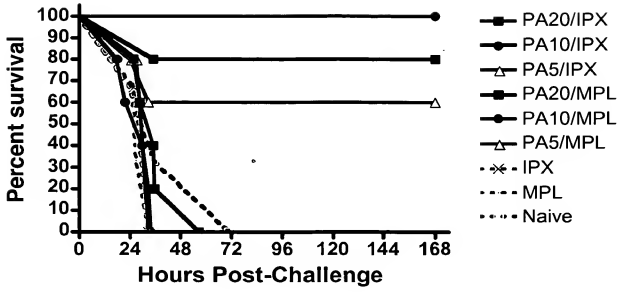
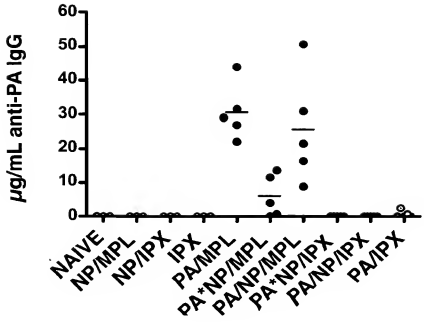

Percent survival
100
90
80
70
60
50
40
30
20
10
0
0
24
48
72
96
120
144
168
Hours Post-Challenge
PA20/IPX
PA10/IPX
PA5/IPX
PA20/MPL
PA10/MPL
PA5/MPL
IPX
MPL
Naive

FIG. 5


μg/mL anti-PA IgG
60
50
40
30
20
10
0
NAIVE
NP/MPL
NP/IPX
IPX
PA/MPL
PA*NP/MPL
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PA*NP/IPX
PA/NP/IPX
PA/IPX

FIG. 6

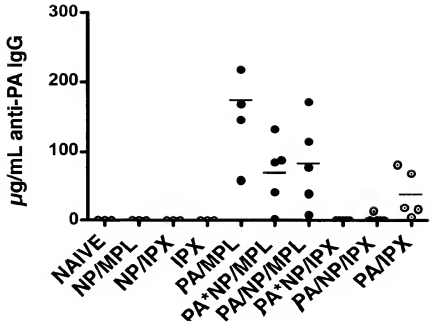

300
200
100
0
μg/mL anti-PA IgG
NAIVE
NP/MPL
NP/IPX
IPX
PA/MPL
PA*NP/MPL
PA/NP/MPL
PA*NP/IPX
PA/NP/IPX
PA/IPX

FIG. 7

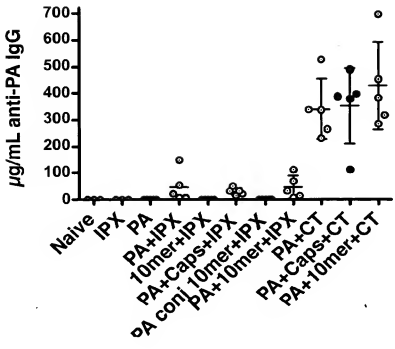
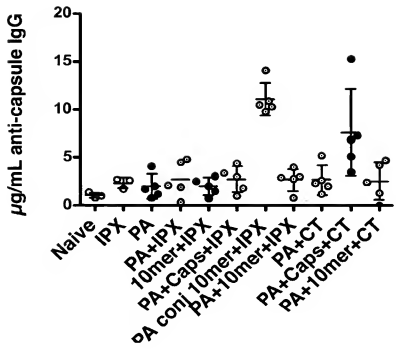

μg/mL anti-PA IgG
700
600
500
400
300
200
100
0
Naive
IPX
PA
PA+IPX
10mer+IPX
PA+Caps+IPX
PA conj 10mer+IPX
PA+10mer+IPX
PA+CT
PA+Caps+CT
PA+10mer+CT

FIG. 8


μg/mL anti-capsule IgG
20
15
10
5
0
Naive
IPX
PA
PA+IPX
10mer+IPX
PA+Caps+IPX
PA conj 10mer+IPX
PA+10mer+IPX
PA+CT
PA+Caps+CT
PA+10mer+CT